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## **Differential investment into testes and sperm production in alternative male reproductive tactics of the African striped mouse (*Rhabdomys pumilio*)**

Schradin, Carsten ; Eder, Susanne ; Müller, Karin

**Abstract:** Males that follow alternative reproductive tactics might differ in their investment into testis development and sperm production. The resource-allocation hypothesis predicts that males following a sneaker tactic should invest more into sperm production than dominant territorial males which should invest more into mate guarding. This hypothesis is supported by studies in species where individual males cannot switch between tactics (fixed tactics). Here we present the first data for a species where males can switch between tactics (plastic tactics). We studied African striped mice (*Rhabdomys pumilio*) in captivity, mimicking three tactics observed in the field: philopatric group-living males, singly-housed males representing roaming males, and group-living breeding males. We measured quantitative and qualitative reproductive traits, as well as serum and testis hormone concentrations. We found no support for the resource-allocation hypothesis, since breeding and singly-housed males invested similarly in testes and sperm. However, philopatric males had significantly smaller testes and epididymides, lower sperm counts, lower testosterone and higher corticosterone levels than males of the two other tactics. Philopatric males did not reach a larger body mass than singly-housed males with well developed reproductive traits, indicating that they did not trade investment in sperm production against growth. Interestingly, testis testosterone concentrations of philopatric males did not differ from those of other males. Our data suggest that philopatric males are reproductively suppressed by the breeding male, but might be ready to increase their serum testosterone levels when social and environmental conditions allow for this physiological switch accompanying the behavioral switch between tactics.

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1 **Differential investment into testes and sperm production in alternative male**  
2 **reproductive tactics of the African striped mouse (*Rhabdomys pumilio*)**

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## ABSTRACT

Males that follow alternative reproductive tactics might differ in their investment into testes development and sperm production. The resource-allocation hypothesis predicts that males following a sneaker tactic should invest more into sperm production than dominant territorial males which should invest more into mate guarding. This hypothesis is supported by studies in species where individual males cannot switch between tactics (fixed tactics). Here we present the first data for a species where males can switch between tactics (plastic tactics). We studied African striped mice (*Rhabdomys pumilio*) in captivity, mimicking three tactics observed in the field: philopatric group-living males, singly-housed males representing roaming males, and group-living breeding males. We measured quantitative and qualitative reproductive traits, as well as serum and testes hormone concentrations. We found no support for the resource-allocation hypothesis, since breeding and singly-housed males invested similarly in testes and sperm. However, philopatric males had significantly smaller testes and epididymides, lower sperm counts, lower testosterone and higher corticosterone levels than males of the two other tactics. Philopatric males did not reach a larger body mass than singly-housed males with well developed reproductive traits, indicating that they did not trade investment in sperm production against growth. Interestingly, testis testosterone concentrations of philopatric males did not differ from those of other males. Our data suggest that philopatric males are reproductively suppressed by the breeding male, but might be ready to increase their serum testosterone levels when social and environmental conditions allow for this physiological switch accompanying the behavioural switch between tactics.

**Keywords** alternative reproductive tactics, mouse sperm, sexual suppression; reproductive suppression; single strategy

## Introduction

The complex process of sperm production is costly in terms of time and energy (White-Cooper et al., 2009). Resource allocation into testes for sperm production and into epididymides for sperm maturation might differ between individuals, especially if intraspecific polymorphism in behavioral tactics to achieve reproductive success exists, referred to as alternative reproductive tactics (ARTs; Pitnick et al., 2009; Taborsky et al., 2008). Within social vertebrates, tactics employed might include being a dominant territorial breeder male (also called bourgeois male), or a sneaker male (also called satellite or roamer male) which solicit matings with females defended by the territorial males (Gross, 1996; Taborsky et al., 2008). As such, ARTs offer a unique opportunity to test the resource-allocation hypothesis, which predicts that males following a sneaker tactic should invest more into sperm production than dominant territorial males which should invest more into mate guarding (Gage et al., 1995; Parker, 1990). In fish for instance, sneaker males can have relatively larger testes (Gage et al., 1995; Oliveira et al., 2005; Stoltz and Neff, 2006).

ARTs can be fixed for life, or they can be plastic whereby individual males might switch between tactics (Gross, 1996). In species with plastic ARTs, sneaker males are less competitive and often smaller and younger than the dominant males, which have the highest fitness. Sneaker males are making the best of a bad job (Dawkins, 1980), following a tactic with low fitness payoffs that is still better than no reproductive success at all. Such plastic ARTs are common in many species of fish (Taborsky, 2008), birds (Krüger, 2008) and mammals (Wolff, 2008). These sneaker males change to the dominant territorial tactic when they grow larger and gain greater resource holding potential. The underlying strategy (decision rule) of such plastic ARTs has been called conditional strategy (Gross, 1996), or more recently a single strategy (because it has one set of decision rules; Schradin and Lindholm, 2011).

It is not clear whether the predictions from the resource-allocation hypothesis (Gage et al., 1995; Parker, 1990) only hold for species with fixed ARTs where the tactic is genetically determined (alternative strategies; Gross, 1996) or also for species with plastic tactics and single strategies (Schradin and Lindholm, 2011). Sneaker males of species with single strategies might not invest more than dominant males in sperm, as they also have to invest in survival to be able to switch to the dominant territorial tactic. Further, if dominant males experience high sperm competition by other males, they might also have to invest highly into sperm production (Pitnick et al., 2009). To our knowledge, all studies on the influence of ARTs on sperm production have been done in species with fixed ARTs, but not in species where males can change tactics.

Two factors have been identified to be likely candidates for the switch to another reproductive tactic (Schradin et al., 2009a): a change in body condition and/or in hormone levels. Both might be triggered by social and environmental conditions (Schradin et al., 2012). Since – irrespective of the resource allocation hypothesis – a good body condition is conducive to produce large numbers of sperm, males differing in body condition are predicted to differ in sperm quantity and/or quality, and this might also depend on age (Pitnick et al., 2009). For species with plastic ARTs, this would predict that dominant males have more and/or better quality sperm than sneaker or younger males, because a better body condition is the prerequisite to reach the dominant bourgeois state. For example in the bank vole (*Myodes glareolus*), dominant males have a greater body mass, larger testes, greater sperm counts, more

95 motile and viable sperm with less sperm abnormalities than subordinate males (Kruczek and  
96 Styra, 2009). Also, in house mice, dominant males have greater numbers of motile sperm  
97 cells than subdominants, though there was no difference in sperm density or velocity (Koyama  
98 and Kamimura, 1998, 2003). Thus, in small mammals, social status can significantly affect  
99 sperm quantity and quality.

100 Significant effects of dominance status on reproductive parameters was demonstrated  
101 in several studies on reproductive suppression of subordinates by dominants in group living  
102 species, especially cooperatively breeding species (Abbott et al., 2003; Clarke et al., 2001;  
103 Molteno and Bennett, 2002; Young et al., 2006). In general, dominant breeders have higher  
104 testosterone levels than sneakers or satellites (Moore et al., 1998; Oliveira et al., 2008), which  
105 might influence testes development. In many group living species, dominant breeders suppress  
106 the reproduction of subordinates by inducing chronically increased glucocorticoid levels in the  
107 subordinates (Reyer et al., 1986; Wingfield and Sapolsky, 2003). In Mongolian gerbil  
108 (*Meriones unguiculatus*), subordinate males typically have small testes and low testosterone  
109 levels, while subordinate males with large testes, viable sperm and high testosterone levels  
110 have a high risk of being expelled from the family group (Scheibler et al., 2006). Subordinate  
111 males of the cooperatively breeding highveld mole-rat (*Cryptomys hottentotus*) have relatively  
112 smaller testes but not lower testosterone levels than dominant breeders, and most subordinate  
113 males have sperm (Rensburg et al., 2003). Thus, reproductive suppression is an alternative to  
114 the resource allocation hypothesis or growth related processes for explaining differences in  
115 reproductive parameters between two ARTs that live in the same social group.

116 While ultimate aspects such as dominance status might affect investment into sperm  
117 production, the question arises as to how differences in sperm production are regulated  
118 proximately. Sperm production (quantity and quality) is positively influenced by testosterone,  
119 while corticosterone might have a negative effect (Kempnaers et al., 2008; Roelants et al.,  
120 2002; Ruwampura et al., 2010; Sung et al., 1997). Testosterone is mainly produced and  
121 secreted by Leydig cells in the testis, promotes spermatogenesis by direct action on Sertoli  
122 cells which are located within the seminiferous tubules of the testes and support and nourish  
123 the adjacent germ cells, leading to the maturation into spermatozoa (Rahman and Christian,  
124 2007). Testosterone is also transferred to the blood circulation to exert its global action on the  
125 male. In species with ARTs, one could expect that males following the tactic that is  
126 characterized by the highest testosterone levels should have a larger quantity and/or better  
127 quality of sperm, while males following a tactic with low testosterone levels should have low  
128 quantity/quality sperm. Thus, for studies on ARTs it is important to measure sperm  
129 characteristics as indicators of reproductive investment and sperm competitiveness, and how  
130 this is influenced by body mass and testosterone.

131 One species with plastic ARTs is the African striped mouse (*Rhabdomys pumilio*) from  
132 the Succulent Karoo semi desert of South Africa (Schradin et al., 2009a). This species is a  
133 group-living solitary forager with communal breeding and helpers at the nest (Schradin and  
134 Pillay, 2004). Groups consist of a single dominant territorial breeding male (the bourgeois  
135 tactic), two to four communally breeding females and their philopatric adult offspring of both  
136 sexes that show allo-parental care (Schradin and Pillay, 2004). Males can follow one of three  
137 ARTs (Schradin, 2008a; Schradin et al., 2009a; Schradin et al., 2009b): (i) philopatric group-  
138 living males, (ii) solitary living roamers, (iii) dominant group-living territorial breeder males.  
139 When reaching adulthood, male striped mice typically remain philopatric in their natal group.

Philopatric males are reproductively suppressed by the breeding male, having high corticosterone and low testosterone levels, small testes and low sperm counts (Schradin et al., 2009b). In the subsequent breeding season, about 50% of philopatric males become roamers first (Schradin et al., 2009a), which is associated with an increase in testosterone and a decrease in corticosterone levels, before becoming territorial breeders and experiencing a decrease in testosterone levels (Schradin and Yuen, 2011). If philopatric males gain sufficient body mass during the dry season and winter, they can directly become territorial breeders at any time of the year, if the breeding position of a neighbouring group becomes vacant; such a switch is associated with a decrease in corticosterone and an increase in testosterone levels (Schradin and Yuen, 2011).

We studied whether investment into sperm production of these ARTs is best explained by the resource-allocation hypothesis or alternatively by sexual suppression. We conducted experiments in captivity, mimicking the situation found in nature. The sons of the breeder males were either left in the families as philopatric males or removed and singly-housed, representing roaming males with high testosterone and low corticosterone levels (Schradin et al., 2009b). We analyzed the inter-ART differences in body mass, hormones, and quantitative and qualitative reproductive traits of captive individuals of striped mice. If the resource allocation hypothesis is important, we expected:

1. Singly-house males (representing roamers) to invest more into sperm production than territorial breeder males.
2. If philopatric males invest less into sperm production, they should increase more in body mass, indicating a trade of between sperm investment *versus* body growth.

If reproductive suppression is important in explaining differences between the ARTs in investment into sperm production, we expected:

1. Singly-house males (representing roamers) to invest more than their philopatric brothers into sperm production, but not more than territorial breeders.
2. If philopatric males are reproductively suppressed, they should not grow faster than their same aged singly-housed brothers, even though they invest less into sperm production.

Finally, we addressed the question whether body condition (measured as body mass and testosterone levels) correlates positively with sperm production (larger testes, better sperm quantity and quality), independent of reproductive tactic. This would give us an indication whether the change of reproductive tactics is a discontinuous switch or a continuous process following a developmental trajectory in each individual, governed by growth (gaining body mass), producing more testosterone and improving development (increasing quantity and quality) of reproductive traits.

## **Materials and Methods**

### *Animal housing*

The colony consisted of descendants from animals originally trapped in 2002 in the Succulent Karoo in South Africa. Animals were kept at the University of Zurich under a 12:12 h light regime. Each of 15 families was housed in two glass tanks 50 x 30 x 30 cm, which were connected to one another by a flexible plastic tube. Additionally, one plastic cage 20 x 13 x 15 cm was connected by another tube, and a water bottle was provided in this cage. Single individuals were kept in one glass tank connected to two plastic cages. All tanks and cages had 5 cm deep wood shavings for bedding. The tanks contained natural branches and each family and singly-housed mouse had one running wheel.

Striped mice in the Succulent Karoo display significant weight fluctuations, gaining weight during spring and losing more than 10% during the following dry season (Schradin and Pillay, 2005a), which can explain why they are prone to extreme obesity in captivity. To avoid obesity and as a means of behavioral enrichment, striped mice were not fed *ad libitum* but three times a day: in the morning they received a seed mix of 4.0 g/individual (guinea pig and hamster food, Haeffliger AG, Herzogenbuchsee, Switzerland), at noon one piece (approx. 1.0 g) of fruit or vegetable per individual and in the afternoon two mealworms per individual. Water was available *ad libitum*.

Animal ethical clearance was provided by the Kantonale Veterinärämte of the Kanton Zürich in Switzerland (ethical clearance number 91/2006).

#### *Experimental procedure*

From every family we used three males for the study: the father (approx. 3 months older than his sons) and two of his sons which were adults and of the same age. Families were kept together until offspring were three weeks old (weaning is on D16; Brooks, 1982). On D21, one male offspring was randomly assigned to be the singly-housed male, his brother the family living philopatric male. Additionally, one male and one female offspring remained in the family such that the philopatric male experienced both a male and a female sibling; all remaining offspring were euthanized. In four of the 15 families, male siblings were not available, so philopatric males thus either had 2 female siblings (2 pairs) or only one female sibling (2 pairs). There was no indication that males who did not experience any male sibling reacted / developed differentially, which is in agreement with a previous study demonstrating that it is the presence of the father, not other family members, that influences the physiology of subordinate males (Schradin et al., 2009b).

For each family, the philopatric male and its singly-housed brother were weighed once a week and their reproductive states were determined as either being non-scrotal (testes inside the body) or being scrotal (testes fully descended), until the week both siblings were recorded as being scrotal (on average after  $5.3 \pm 1.2$  weeks). When the male offspring were 9-10 weeks old the experiment was ended. At this age, all males were fully scrotal. This represented the age at which males would have dispersed and become solitary roamers under field conditions of very low population density (Schoepf and Schradin, 2012; Schradin, 2005). Their body mass (to the closest 0.1 g) was determined before they were anaesthetized and a blood sample of 300  $\mu$ l was taken using sublingual blood sampling (Heimann et al., 2009). The blood was allowed to clot for 1.5 h at ambient temperature and was then centrifuged for 10 min at 10000 x g. The resulting serum was pipetted and frozen in aliquots. Males were euthanized immediately after blood sampling and the testes and caudae epididymides were removed. The

same morning, the father of these males was also sampled for blood, euthanized, and his testes were dissected. Testes (and the surrounding tissue) were kept at 4°C in a plastic bag and couriered in an isolated transport box (MTG, Germany) at 4-8°C to the Leibniz Institute for Zoo and Wildlife Research (Berlin, Germany) for further analyses. Further preparation of testes occurred 48-58 h after euthanasia, until which time testes were kept in a plastic bag at 4°C.

### *Quantitative parameters*

All chemicals for the laboratory analyses were purchased from Invitrogen (Germany) if not stated otherwise. Both testes and epididymides of each individual were freed from surrounding tissue and blood vessels. Each testis was dissected from the epididymis and testes and epididymides mass (without the vas deferens) from both sides were measured. Then, the caput and cauda epididymides were separated as shown in figure 1 and weighed again separately. The length (l) and width (w) of each testis was also measured and the volume was calculated by the following formula:  $V = 4/3 \pi * w^2 * l$ .

To determine the number of testicular spermatozoa, testis parenchyma from the outer third of testis (avoiding the rete testis region) was cut with a sharp razor blade into small pieces. 0.1 g testis parenchyma was weighed, minced and suspended in 2 ml M199 (a complex cell culture containing Hepes buffer to avoid a fast change of its pH-value on air; Sigma M 7528) supplemented with 0.4% (w/v) BSA (Merck, Germany, fraction V, K32491618-406) while carefully pressing through a 28 µm nylon mesh. After appropriate dilution in water, the number of sperm was counted twice in a haemocytometer and the means were expressed as sperm concentration (per g testis) and total sperm number in testis (sperm concentration x testis mass) as well.

Cell cycle stages in the testes give a measurement of gonadal activity, i.e. whether and to what extent meiosis is taking place and spermatozoa are produced. Haploid signals come from post-meiotic germ cell stages like spermatids and sperm cells, diploid signals from spermatogonia, secondary spermatocytes and somatic testicular cells, tetraploid signals mainly derive from G2/M phase of cell cycle in meiotic primary spermatocytes but also in mitotic spermatogonia.

A high proportion of haploid nuclei indicates a high meiotic activity and thus sperm production. A low ratio of diploid to tetraploid signals or a high ratio of haploid to diploid signals is indicative of the occurrence of meiosis. The ratio of haploid to tetraploid nuclei is a measure of the meiotic yield from primary spermatocytes to the haploid “product”.

For the analysis of cell cycle stages in the testes, testis parenchyma was frozen at -20°C until use. After thawing, testicular cells were dispersed and flow cytometric DNA analysis was applied according to Blottner et al. (1998): 0.1 g of testis parenchyma was finely minced in 1 ml 100 mM citric acid containing 0.5% (v/v) Tween 20 and agitated for 20 min at room temperature. The DNA was stained by adding 4 ml of a 400 mM Na<sub>2</sub>HPO<sub>4</sub> solution containing 5 µM 4',6-diamidino-2-phenylindol (DAPI) for 10 min in the dark. Measurements were performed on a PAS III flowcytometer (Partec, Germany) equipped with a mercury lamp and an appropriate filter set (excitation: 360 nm, emission: 420 nm). For each sample, 15000



cells were counted and the histograms were analysed for the proportions of cells in each peak by the FlowMax software (Partec, Germany).

Both parts of epididymides were finely minced by scissors in 200-1500  $\mu$ l (depending on the epididymis size) M199-k composed of M199 (Sigma M 7528) supplemented with 1 mM sodium pyruvate, 14 mM sodium lactate and 0.4% BSA. Each cell suspension was filtered through a 30  $\mu$ m cell filter (Partec, Germany). The sperm concentration was counted after appropriate dilution with water in a haemocytometer.

#### *Qualitative sperm parameters*

Sperm morphology, sperm motility and mitochondrial status were analyzed to give basic information about whether sperm were functional. This was done before (caput sperm) and after (cauda sperm) maturation in the epididymis. Qualitative parameters refer to all parameters describing the sperm quality, which can be both categorical (e.g. % of sperm with a minimum distance passed during measurement) or non-categorical parameters (e.g. mean velocity of this sperm population, which can be measured quantitatively).

For evaluating the morphology of spermatozoa, 10  $\mu$ l filtered sperm suspension (from the refrigerated testes) were fixed with 90  $\mu$ l 0.5% formaldehyde in PBS Dulbecco (Sigma D 8537) for 1 h at room temperature and stored at 4°C until observation. Droplets of 8  $\mu$ l on a slide were covered with a coverslip and counted for intactness of heads and tails under a microscope (Leica, Germany) with phase contrast optics and a 1000 x magnification using oil immersion.

For evaluating sperm motility, the filtered sperm suspension was diluted with M199-k to about  $4-8 \times 10^6$  sperm cells / ml and 100  $\mu$ l were incubated in a closed Eppendorf tube at 38°C. After 5 min incubation, 5  $\mu$ l sperm suspension were pipetted in a pre-warmed Makler chamber (Sefi-Medical Instruments, Israel) and motility was examined by computer-assisted sperm analysis system SpermVision (Minitüb, Germany) equipped with a Nikon microscope, dark field optics (Nikon, Japan) and a video camera TM-6760CL (JAI Pulnix, Germany). The temperature of the microscope stage was maintained at 38°C. For each field, 30 pictures were recorded at 60 Hz, and 8 fields with 30 to 50 sperm cells per field were evaluated per sample. The percentage of motile and progressively motile spermatozoa as well as the curvilinear velocity (VCL) of progressively motile spermatozoa (cells that have passed a distance  $\geq 5\mu$ m during the 0.5 seconds of recording) was evaluated.

To assess the number of sperm cells with active mitochondria, the filtered sperm suspension was diluted with M199-k to  $1-2 \times 10^6$  sperm cells in 250  $\mu$ l M199-k and 1  $\mu$ l rhodamine 123 (R-302) from a stock (50  $\mu$ g/ml) as well as 2.5  $\mu$ l propidium iodide (P 3566) from a stock (1 mg/ml) were added. After 20 min incubation at 37°C in the dark, 10-40  $\mu$ l were measured in 1 ml pre-warmed M199-k on a PAS III flowcytometer (Partec, Germany) equipped with an 200 mW Argon laser (excitation: 488 nm). Rhodamine 123 accumulates in active mitochondria membranes and the resulting green fluorescence was recorded using a band pass (500-560 nm). Propidium iodide stains the DNA of non-viable sperm cells and the resulting red fluorescence was recorded using a long pass ( $> 610$  nm). For each sample, 15000 events were counted. Sperm cells were discriminated from contaminations in a forward-sideward scatterplot. After defining the sperm cell signals by gating, the percentage of the

sperm population with active mitochondria was analyzed in the green versus red fluorescence dotplot by the FlowMax software (Partec, Germany).

#### *Hormone assays*

Serum samples were analyzed for testosterone and corticosterone in appropriate dilutions using commercial kits from IBL Hamburg (for validation for the study species see (Schradin, 2008b). For corticosterone, intra-assay and inter-assay variability were 5.3% and 11.3%. For testosterone, intra-assay and inter-assay variability were 3.3% and 11.8%, respectively.

We determined testicular testosterone levels according to Blottner et al. (1998). Two portions of 0.1 g testis parenchyma (0.1 g per side, see below) were frozen per individual at -20°C. The parenchyma was lyophilized and testosterone was extracted two times with 1.5 ml 90% methanol for 30 min. The extract was pooled and diluted 1:1 (v/v) with water and duplicates of 20 µl were analyzed. The enzyme immunoassay used a rabbit polyclonal antibody against testosterone-11-HS-BSA and testosterone-3-CMO-peroxidase as enzyme conjugate. The intra-assay and inter-assay variability were 8.9% and 12.3%, respectively.

#### *Statistical analysis*

From the 15 families, three were excluded from analysis because in one family the philopatric male was attacked and had to be removed from the family group. In another family, the singly-housed male died of unknown reasons when 8 weeks old, and in the third family, the breeding male was attacked and removed. Thus, effective sample size is  $n = 12$  families (36 males). In some cases, we could not obtain measurements for all individuals, further reducing sample size (see Tab. 1).

The software Instat (GraphPad) was used. Data within families were compared using repeated measures ANOVA followed by the Tukey-Kramer Multiple Comparison test (q). If data were not normally distributed, we used the non-parametric Friedman ANOVA. Percentage data, though bounded by 0.0 and 100.0%, were treated like normally distributed data if their distribution did not differ significantly from a normal distribution. Comparisons between brother pairs (family vs. single kept males) were done using the Wilcoxon matched pair rank sign test (T) when data were not normally distributed, otherwise we used a paired t-test. Data are presented as mean  $\pm$  standard deviation. Pearson product moment correlations were performed by using the software SigmaStat3.1 (Systat software, Inc.) and the correlation coefficients (r) and p-values are given.

When multiple comparisons were made for the same hypothesis, we applied sequential Bonferroni correction (Rice, 1989). For quantitative measurements, we applied this method for the nine measures of testis and epididymis mass / volume, the four measurements of numbers of sperm, the three measurements of meiosis, the four measurements of sperm motility, the two measurements of intact sperm in the cauda and caput of the epididymis (Tab. 1). We report the original p-values and indicate where these would not have remained significant after Bonferroni correction.

## Results

### *Influence of transport on the samples*

We observed good motility of caput epididymal spermatozoa after transport of the testes from Zurich to Berlin. However, for most qualitative sperm parameters, we noted a reduction of their levels from caput to cauda region of epididymis (Tab. 1). Correlations among qualitative sperm parameters revealed that samples with more motile sperm or more sperm cells with active mitochondria unexpectedly showed more flagellum defects. Further, the loss of active mitochondria between caput and cauda epididymis was lowest in those samples with many flagellum defects. Those flagellum defects can be predominantly characterized by a pattern of coiled tails which has been described to be typical for immature spermatozoa in boar (*Sus scrofa*; Briz et al., 1995). Sperm cells lose the flexibility to fold their tail at the midpiece during epididymal transit when the mitochondrial sheath and the outer dense fibers change properties. This suggests that the more mature sperm cells from the cauda epididymis are more susceptible to the long transport of testes and epididymides whereas the more immature samples of caput retain their viability better. As this was the same for males of all three tactics, it does not influence our statistical comparisons.

### *Comparison between breeders and philopatric males*

All quantitative reproductive parameters had higher mean values in breeder males than in philopatric males and most of these differences were significant (Tab. 1). If quantitative parameters were standardized by body or testis mass, some comparisons were no longer statistically significant. Breeder males had greater total number of sperm than their philopatric sons, but not relative to body or testis mass. Parameters of spermatogenesis were on average higher in breeders than in their philopatric sons, but this was not significant after Bonferroni correction (Tab. 1).

Several mean values of qualitative reproductive traits for epididymal sperm cells were higher in breeders than in philopatric males. However, only three differences were significant: the difference in the percentage of (i) progressively motile and (ii) of morphologically intact cauda sperm cells and the (iii) difference in the percentage of caput sperm with active mitochondria.

Serum corticosterone did not differ between the two groups whereas serum testosterone was significantly lower in philopatric males compared to breeders. Testis testosterone and testosterone per g tissue did not differ (Tab. 1).

### *Comparison between singles and philopatric males*

Singly-housed males became scrotal at an earlier age than philopatric males ( $4.1 \pm 0.3$  weeks vs.  $5.3 \pm 1.2$  weeks;  $T = 0$ ,  $N = 12$ ,  $p = 0.008$ , Wilcoxon Test) and at a lower body mass ( $31.0 \pm 4.8$  g vs.  $35.2 \pm 7.9$ g;  $t_{11} = 2.624$ ,  $p = 0.02$ ). Single and philopatric males had a similar body mass at the end of the experiment (Tab. 1). Single males had higher mean values in their quantitative parameters than their philopatric brothers and many of these differences were significant, especially absolute and relative testis and epididymis mass as well as number of sperm (Tab. 1). For qualitative sperm parameters we found singly-housed males to have a

significantly higher percentage of morphologically intact sperm and higher percentage of sperm with active mitochondria.

Singles had significantly lower corticosterone levels than philopatric males. Serum testosterone levels in singles were four times higher than the values of their philopatric brothers, whereas the testicular testosterone did not differ significantly (Tab. 1).

#### *Comparison between breeders and singles*

Breeders had a significantly greater body mass, testis mass, testis volume, cauda and caput epididymis mass than their singly-housed sons. Quantitative and qualitative sperm parameters were similar between both male groups (Tab. 1). The serum corticosterone levels were significantly lower in singles than in breeders whereas the testosterone concentrations in serum and testis did not differ between the two types of males.

#### *Body condition and reproductive traits*

Body mass was correlated with testis mass (Fig. 3A) and sperm quantity in the testes when the individuals from all three ARTs were considered, but not for any of the three male ARTs alone (Tab. 2). For the most accurate measurement of sperm quality we used the percentage of progressively motile sperm in the cauda epididymis, which was significantly correlated with body mass when data from all individuals of the three ARTs were considered, as well as for single-kept males alone, but not for philopatric or breeding males (Fig. 3B).

Serum testosterone levels were not significantly correlated with testis mass.. Serum testosterone levels were correlated with sperm quantity in the testes when the individuals from all three ARTs were considered, but not for any of the three male ARTs alone (Tab. 2). The percentage of progressively motile sperm in the cauda epididymis (indicating sperm quality) did not correlate significantly with testosterone levels over all males, but significantly and positive within single-kept males.

Over all males, serum testosterone levels correlated significantly with testis testosterone concentration ( $r=0.51$ ,  $p<0.01$ , Bonferroni corrected  $p<0.05$ ; Fig. 4A), and the total testis testosterone production (testis testosterone x testis mass;  $r=0.51$ ,  $p<0.01$ , Bonferroni corrected  $p<0.05$ ; Fig. 4B). These relationships were not found within of any of the three tactics with the exception of a significant correlation between serum and testis testosterone in singly-housed males ( $r=0.71$ ,  $p=0.01$ , Bonferroni corrected  $p<0.05$ ).

## **Discussion**

Male striped mice kept under different social conditions representing three plastic alternative reproductive tactics (ARTs) differed in their development of testes and investment into sperm production. Singly-housed males resembled their fathers, the dominant breeding

males, in most parameters, even though fathers were much older, indicating that age had no effect in our study. Further, there was no indication that singly-housed males, representing the roaming tactic in nature, invest more in sperm production than breeding males. On the other hand, philopatric males living with their natal family including their father had much smaller testes and lower sperm counts, even though they were reproductively mature, i.e. having fully descended testes and functional sperm. There was no indication of a trade-off between investment in sperm production *versus* growth, as philopatric males did not grow faster than singly-housed males; instead philopatric males became scrotal (i.e. went through puberty) at a later age and greater body mass. Thus, the resource-allocation hypothesis was not supported, indicating that the most parsimonious explanation for our results is instead reproductive suppression.

#### *Did singly-housed males represent a good model for roamers?*

In contrast to free living roamers, our singly-housed males did not meet any breeding females during the course of the study. However, under free-living conditions, striped mouse roamers do not have many sexual encounters with breeding females and many roamers have no reproductive success at all (Schradin and Lindholm, 2011). Further, in muroid rodents like the striped mouse it has been shown that sexual stimulation of males by females occurs mainly via olfactory cues (Pillay, 2000), and our singly-housed males were housed next to breeding females and thus exposed to some of their olfactory cues. Keeping striped mouse males in social isolation for several weeks might have influenced their physiology in an artificial way. We think this was not the case, as (i) they still had olfactory contact with other mice, (ii) we kept them under highly enriched conditions such that they did not show any stereotypic behaviour that is typical for group-house striped mice kept in standard cages (Schradin, unpubl. data), and (iii) the observed changes in hormone levels were similar to the changes observed under field conditions (Schradin & Yuen, 2011). Hormonally, singly-housed males differed from family housed males in the same way as roamers differ from philopatric males (Schradin et al., 2009a): they had higher testosterone but lower corticosterone levels, and were scrotal. Behaviourally, singly-housed males represented roamers in the most important part, i.e. they lived solitarily. However, they could not roam over wide areas. This might help explain why they did not show one important physiological trait of roamers: their testosterone levels were not higher than those of breeders, as has been reported from the field (Schradin et al., 2009a; Schradin and Yuen, 2011). High testosterone levels in free ranging roamers might be a consequence of roaming rather than its cause; for example encounters with other males might lead to increased testosterone secretion, as predicted by the challenge hypothesis (Wingfield et al., 1990). While our model could not mimic all aspects of the life of a free ranging roaming male, it covered many important aspects and led to the predicted changes in hormone levels.

#### *Does sperm production depend on body condition?*

In the field, striped mouse males of the three ARTs differ in body mass, with breeders being the heaviest (and oldest), philopatric males the smallest (and youngest), and roamers intermediate in body mass and age (Schradin et al., 2009a). In our captive study, breeders were also the heaviest and oldest males, but philopatric males did not differ significantly from their

singly-housed brothers. Body mass is often used as a measurement of body condition, and males with good body condition might be able to invest more in sperm production (Pitnick et al., 2009). Accordingly, we found that larger males had larger testes and more sperm when comparing over all males. However, within each tactic we did not find this relationship, i.e. larger philopatric males / single males / breeders did not have larger testes than smaller philopatric males / single males / breeders (Tab. 2). Our results for an influence of testosterone on sperm quantity and quality were similar. Over all males, we found a positive correlation between serum testosterone levels and number of sperm, but not for any of the tactics alone. This indicates that our significant results for all males represent pseudo-correlations arising from class differences and allometric relationships: breeders are larger and have larger testes and more sperm than philopatric males. Similar results were found in cooperatively breeding common marmosets (*Callithrix jacchus*), where testes mass correlates with body mass for all males, but not within male categories: dominant breeding males have larger testes than smaller adult subordinate helper males (Araujo and Sousa, 2008).

The fertilizing efficiency of a male depends on the quantity of functionally competent sperm (Aitken, 2006; Rodriguez-Martinez and Barth, 2007). The qualitative and most quantitative reproductive traits were similar between larger breeding and smaller singly-housed striped mouse males. Therefore, it is not simply age or body mass that triggers sperm production and sperm quality. Compared to philopatric males the singly-housed males had larger testes, higher serum testosterone levels, and more sperm which were more often intact. These differences between singly-housed and philopatric males cannot be explained by age or body mass. Investment into sperm production and testes development does not seem to be strongly influenced by body mass or age, but by the reproductive tactic of a male. Similar results were found in a field study measuring reproductive success in striped mice: reproductive success depended strongly on tactic, which itself was correlated with body mass, but within tactics, body mass did not influence reproductive success (Schradin and Lindholm, 2011). These results from different studies are in agreement with the hypothesis that the three ARTs represent real tactics that differ categorically in physiological mechanisms, reproductive traits, behavior and fitness.

#### *Reproductive suppression but no trade-off between investment into sperm versus growth*

Philopatric males, even though they were scrotal, had lower testosterone levels, smaller testes and lower sperm counts than their same aged singly-housed brothers of similar body mass. Philopatric males thus showed clear signs of being reproductively suppressed, supporting a previous study (Schradin et al., 2009b). Compared to their singly-housed brothers, they had higher corticosterone levels, which is regarded as an indicator of physiological reproductive suppression (Reyer et al., 1986; Wingfield and Sapolsky, 2003). However and in contrast to field data (Schradin, 2008b), in our present study, both breeders and philopatric males had similar corticosterone levels and higher corticosterone levels than singly-housed males. In the field, we measured corticosterone levels of 2000 ng/ml in philopatric males (4.7 ± 1.9 months old; Schradin et al., 2009a), and in another captive study with the same colony 1053 ng/ml in five weeks old philopatric males that just became scrotal (Schradin et al., 2009b), both values being much higher than in the present study (278 ng/ml when 10 weeks old). High corticosterone levels might be one of several mechanisms of reproductive suppression (Schradin et al., 2009b), but in our present study, the low

testosterone levels of philopatric males cannot be explained by simultaneously high corticosterone levels, as otherwise breeders would also be expected to have low testosterone levels. Alternatively, our data might represent the last physiological signals of previously high corticosterone levels.

We found that testis and epididymis mass parameters were lowest in philopatric males. Also, there was some evidence that sperm of philopatric males had lower maturity and lower functionality, especially lower progressive cauda motility, fewer caput sperm with active mitochondria, and fewer morphologically intact sperm. The ploidy analysis of testicular cells revealed a low but detectable spermatogenetic activity in philopatric males. These results indicate that reproductive suppression's primary effect is on the amount of reproductive tissue that produces sperm and only secondarily on sperm functionality. It can be expected that these effects would be stronger if reproductive suppression is stronger, e.g. in younger males, or if additional stressors occur under field conditions (low food availability, encounters with territorial neighbors; Schradin et al., 2009b). Importantly, our data indicate that while philopatric males have lower fertility, they are not necessarily infertile. This corresponds to field data, where philopatric males have very low reproductive success, but some of them sire offspring with females from neighboring groups (Schradin and Lindholm, 2011).

*Does the resource-allocation hypothesis hold for species with plastic ARTs?*

Philopatric males became scrotal at an older age and greater body mass than their singly-housed brothers, but philopatric males did not reach a larger body mass at the end of the study than their singly-housed brothers. Thus, the reduced investment into testes and sperm production by philopatric males indicates reproductive suppression rather than a trade-off between growth *versus* reproduction. Compared to breeding males, singly-housed males did not invest more in testes or sperm production as predicted by the resource-allocation hypothesis, nor did sperm functionality differ between breeders and singly-housed males. In sum, we found no support for the resource-allocation hypothesis.

*Is the change of reproductive tactics a continuous process or a discontinuous switch?*

Philopatric males did not have lower testis testosterone concentrations compared to breeders. This indicates that testosterone production per gram testes parenchyma (philopatric males *vs.* breeders: 83.3 *vs.* 85.7 ng/g tissue) might be conserved in the same way as the sperm production rate per gram parenchyma is constant in mammals (Moller, 1989). We hypothesize that philopatric males are primed to increase their serum testosterone levels very quickly when social and environmental conditions allow for this, e.g. if they disperse and become roamers or if a breeding position becomes vacant in a neighbouring territory. Increased testosterone secretion into the blood could then be maintained by increasing testis size.

In field studies, the switch between tactics is determined by behavioral switches, especially from group- to solitary-living back to group-living (i.e. philopatric to roamer to breeder; Schradin et al., 2012). Our present study indicates that these switches between tactics are accompanied by physiological switches, such as changes in hormone levels, testes development and functionality. In nature, increases in testosterone levels and testes development are likely to occur before philopatric males decide to leave their natal group to

become roamers and try to find a breeding position in another group. All three ARTs are characterized by a similar testosterone concentration in testes that ensures basic sperm production, even in philopatric males. In the cooperatively breeding cichlid *Neolamprologus pulcher*, non-reproducing males living in a school have high testosterone levels, which might enable them to rapidly switch to breeding condition once they become territorial breeders (Bender et al., 2008). In striped mice, testis size and sperm production may be increased by social and environmental factors, for example the presence of reproductively active females (Amstislavskaya and Popova, 2004; Macrides et al., 1975), high food availability and the absence of a dominant male (Schradin et al., 2009b).

If the change in reproductive tactics is a continuous process depending on the growth of individuals, we would have expected correlations between body mass and reproductive traits within the three male tactics studied. However, within each tactic neither body mass nor testosterone levels determined reproductive traits. Instead, we often found correlations between body mass and reproductive traits when we considered all males in the analysis, representing statistical pseudocorrelations that were due to differences in body mass between the three categorical tactics. Our study indicates that the three tactics represent three clear categories and not simply three points on a developmental trajectory. Thus, the striped mouse fits the definition of a species with discontinuous reproductive traits in males following different tactics, one of the main characteristics of real ARTs (Taborsky et al., 2008).

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**Table 1:** Body mass, sperm traits and hormones for individuals of the three ARTs studied in a striped mouse colony, where two sons of each breeder male were randomly attributed to a philopatric life in the family or a single life without any other individuals. The number of families studied, mean (STDEV),  $F / \chi^2$  and overall p-values are provided. Significant pair-wise comparisons and their direction are indicated shaded in the last three columns, with dark grey for differences in the direction “>” and light grey for differences in the direction “<” (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; \*\*\*\*:  $p < 0.0001$ ). p-values that would not remain significant after sequential Bonferroni correction for multiple tests for the same hypothesis are marked by <sup>sB</sup> and are not shaded.

Type of traits		n	Breeders	Philopatrics	Singles	F, p	Breeders-Philopatrics	Breeders-Singles	Singles-Philopatrics	
Body mass		[g]	12	71.4 (9.7)	49.6 (4.5)	49.6 (6.5)	F <sub>2,22</sub> =39.086, ****	>****	>****	=
Quantitative reproductive traits	Testis mass	[g]	12	1.28 (0.26)	0.71 (0.24)	1.00 (0.25)	F <sub>2,22</sub> =15.885, ****	>***	>*	>*
	Testis mass / body mass	[%]	12	1.82 (0.44)	1.42 (0.45)	2.04 (0.52)	F <sub>2,22</sub> =5.016, * sB	=	=	>*
	Testis volume	[mm <sup>3</sup> ]	12	12 130 (2 280)	6 338 (1 842)	8 876 (2 140)	F <sub>2,22</sub> =23.709, ****	>***	>***	>*
	Epididymis mass	[g]	12	0.301 (0.043)	0.120 (0.055)	0.190 (0.057)	F <sub>2,22</sub> =36.599, ****	>***	>***	>***
	Epididymis mass / body mass	[%]	12	0.43 (0.07)	0.24 (0.12)	0.39 (0.12)	F <sub>2,22</sub> =10.480, ***	>***	=	>***
	Cauda epid. mass	[g]	12	0.117 (0.033)	0.040 (0.019)	0.074 (0.026)	F <sub>2,22</sub> =30.452, ****	>***	>***	>***
	Cauda epid. mass / body mass	[%]	12	0.16 (0.04)	0.08 (0.04)	0.15 (0.05)	F <sub>2,22</sub> =14.999, ****	>***	=	>***
	Caput epid. mass	[g]	6	0.146 (0.034)	0.068 (0.029)	0.112 (0.025)	F <sub>2,10</sub> =11.028, **	>***	=	=
	Caput epid. mass / body mass	[%]	6	0.20 (0.05)	0.14 (0.06)	0.22 (0.06)	F <sub>2,10</sub> =2.357, p=0.15	=	=	=
	Sperm number in testis	[x 10 <sup>6</sup> ]	12	168 (55)	80 (57)	139 (52)	F <sub>2,22</sub> =10.581, **	>***	=	>*
	Sperm number in testis / Testis mass	[x 10 <sup>6</sup> /g]	12	131 (39)	101 (47)	138 (40)	F <sub>2,22</sub> =4.450, *	=	=	>*
	Sperm number in testis / Body mass	[x 10 <sup>6</sup> /g]	12	2.4 (0.9)	1.6 (1.1)	2.8 (0.9)	χ <sup>2</sup> =9.500, **	=	=	>***
	Sperm number in cauda	[x 10 <sup>6</sup> ]	12	209	35	161	F <sub>2,22</sub> =5.111,	>*	=	=

			epididymis	(153)	(53)	(152)	*			
	%haploid / %diploid cells in testis		12	7.8 (1.4)	5.6 (2.5)	7.2 (2.2)	$F_{2,22}=4.761$ , *sB	>*	=	=
	%haploid / %tetraploid cells in testis		12	7.5 (2.6)	7.3 (3.6)	7.1 (2.6)	$\chi^2=1.167$ , p=0.558	=	=	=
	%diploid / %tetraploid cells in testis		12	0.9 (0.2)	1.4 (0.5)	1.1 (0.4)	$\chi^2=8.667$ , *sB	<*	=	=
Qualitative reproductive traits	<i>Cauda epididymal sperm</i>									
	Motile sperm	[%]	11	18.11 (8.26)	12.31 (7.66)	14.98 (6.70)	$F_{2,20}=2.218$ , p=0.13	=	=	=
	Progressively motile sperm	[%]	11	7.21 (4.11)	2.05 (2.21)	5.59 (4.64)	$F_{2,20}=7.000$ , **	>*	=	=
	Velocity progressively motile sperm	[μm/s]	7	143 (52)	148 (48)	163 (29)	$F_{2,12}=0.331$ , p=0.72	=	=	=
	Sperm with active mitochondria	[%]	10	28.70 (13.28)	18.97 (12.63)	23.02 (8.94)	$F_{2,18}=2.259$ , p=0.13	=	=	=
	Morphologically intact sperm	[%]	12	65.00 (12.37)	53.83 (17.56)	70.92 (11.92)	$F_{2,22}=8.426$ , **	>*	=	>**
	Sperm with defect flagellum	[%]	12	25.25 (11.10)	33.17 (18.73)	22.25 (10.09)	$F_{2,22}=5.395$ , **	=	=	<*
	<i>Caput epididymal sperm</i>									
	Motile sperm	[%]	10	35.91 (10.33)	35.64 (12.94)	39.68 (9.75)	$F_{2,18}=0.818$ , p=0.46	=	=	=
	Progressively motile sperm	[%]	10	13.85 (7.33)	10.23 (10.36)	16.21 (7.89)	$F_{2,18}=1.914$ , p=0.18	=	=	=
	Velocity progressively motile sperm	[μm/s]	7	153 (25)	164 (50)	154 (20)	$F_{2,12}=0.219$ , p=0.81	=	=	=
	Sperm with active mitochondria	[%]	9	47.47 (11.18)	34.12 (12.42)	47.39 (11.92)	$F_{2,16}=6.799$ , **	>*	=	>*
Hormones	Serum corticosterone	[ng/ml]	12	251 (118)	279 (179)	132 (87)	$F_{2,22}=6.018$ , **	=	>*	<**
	Serum testosterone	[ng/ml]	12	4.84 (3.75)	1.15 (2.01)	4.95 (4.88)	$F_{2,22}=5.091$ , *	>*	=	>*
	Testis testosterone	[ng/g]	11	85.74 (45.46)	83.26 (74.58)	109.13 (83.18)	$F_{2,20}=0.4913$ , p=0.62	=	=	=

	Testis testosterone total	[ng]	11	113.2 (67.0)	67.5 (71.4)	108.2 (95.8)	$F_{2,20}=0.1.226,$ $p=0.32$	=	=	=
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**Table 2:** Pearson product moment correlations between parameters of body condition (body mass and testosterone levels) and parameters of sperm production. Original p-values are presented. Bonferroni adjusted p-values are presented for significant results with sequential correction for each hypothesis (4 comparisons in each row), and presented as  $p^*$ .

<i>Key parameter</i>	<i>Correlated with</i>	<i>Over all</i>	<i>Breeders</i>	<i>Philopatrics</i>	<i>Singles</i>
<b>Body mass</b>	Testis mass	<b>0.544 / &lt; 0.001</b> <b><math>p^* &lt; 0.01</math></b>	-0.043 / 0.895	0.365 / 0.244	0.268 / 0.399
	Sperm number in testis	<b>0.412 / 0.012</b> <b><math>p^* &lt; 0.05</math></b>	-0.219 / 0.494	0.288 / 0.363	0.568 / 0.054
	Cauda progr. motile sperm	<b>0.468 / 0.006</b> <b><math>p^* &lt; 0.05</math></b>	0.215 / 0.525	-0.618 / 0.043 $p = \text{n.s.}$	<b>0.744 / 0.009</b> <b><math>p^* &lt; 0.05</math></b>
<b>Serum testosterone</b>	Testis mass	0.393 / 0.018 $p = 0.07$	0.098 / 0.762	0.361 / 0.248	0.231 / 0.469
	Sperm number in testis	<b>0.442 / 0.007</b> <b><math>p^* &lt; 0.05</math></b>	0.048 / 0.881	0.408 / 0.188	0.453 / 0.139
	Cauda progr. motile sperm	0.266 / 0.171	-0.004 / 0.991	-0.226 / 0.626	<b>0.765 / 0.010</b> <b><math>p^* &lt; 0.05</math></b>

## Figure legends

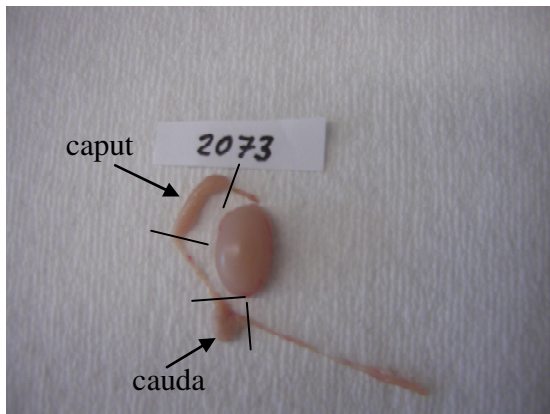
**Figure 1:** Testis and epididymis of a striped mouse male after preparation. For sperm collection, caput and cauda epididymis (arrows) were separated as indicated by the lines.

**Figure 2:** Indication of reproductive suppression in philopatric males, which had smaller epididymis (% of body mass; N=12 for each male classe), less sperm in the cauda epididymis (measured in billion; N=12 for each male class) and less sperm active mitochondria in the caput epididymis (rel. amount of sperm with active mitochondria, 0.47 represents 47%; N=12 for each male class). “a” and “b” superscripts indicate significant pair-wise differences (post-tests) to categories with another subscript. Mean and standard deviation are shown.

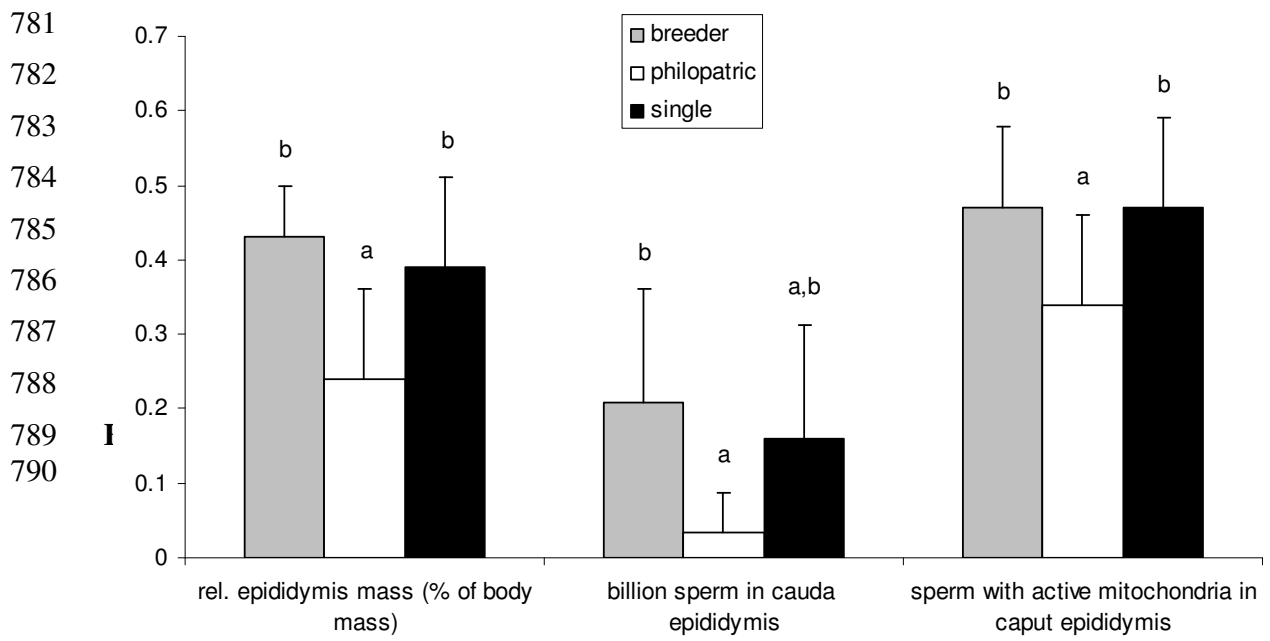
**Figure 3:** The relationship between body mass of striped mouse individuals and (A) testis mass ( $R = 0.544 / p < 0.001$  over all individuals) as well as (B) the percentage of progressively motile sperm cells in the cauda epididymis ( $R = 0.468 / p = 0.006$  for all individuals and  $R = 0.744 / p = 0.009$  within the group of singles; see also Tab. 2).

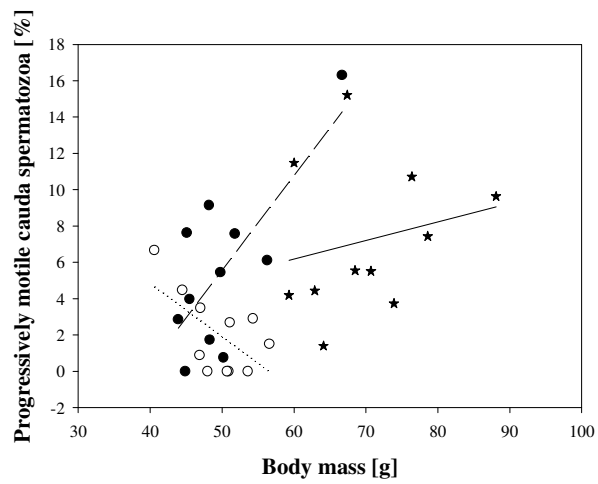
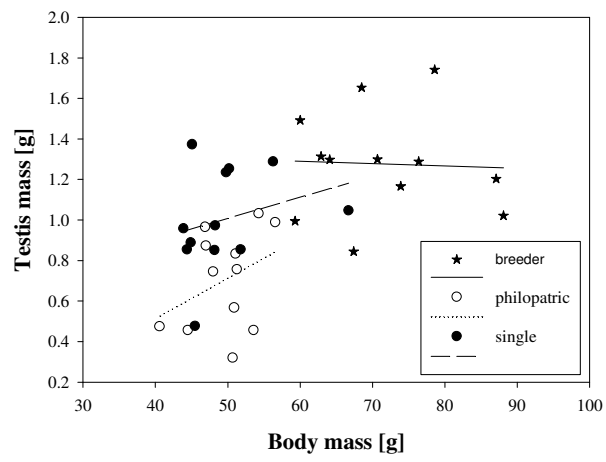
**Figure 4:** The relationship between serum and testis testosterone in individuals of the striped mouse which were attributed to different alternative reproductive tactics. (A) Testis testosterone per g testes ( $R = 0.511 / p = 0.002$  over all individuals and  $R = 0.714 / p = 0.009$  within the group of singles; see also Tab. 2). (B) Total testis testosterone (testis testosterone concentration x testis mass;  $R = 0.621 / p < 0.0001$  over all individuals and  $R = 0.802 / p = 0.003$  within the group of singles).





**Figure 1**





**Figure 3**

